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**14. ABSTRACT**

Modern breast cancer therapies utilize non-specific approaches to kill or remove cancerous cells, inflicting significant collateral damage to healthy cells. In response to the need for highly targeted detection and destruction of cancerous cells, we propose to implement multi-input genetic circuits that act as cell state classifiers based on mRNA or microRNA expression profiling.

The mRNA sensing project is focused on the MCF-7 breast adenocarcinoma cell line. MCF-7 cells overexpress Gata3, NPY1R and TFF1 mRNA relative to healthy cells. Based on our bioinformatics analysis, taking into account the three biomarkers allows for dramatically improved specificity in comparison to targeting single genes. We therefore design a three-input AND gate that triggers a response only when all three biomarkers are expressed above a defined threshold.

In second approach we implement transcriptional/post-transcriptional regulatory circuit that senses expression levels of a customizable set of endogenous microRNAs and computes whether to trigger a response if the expression levels match a pre-determined profile of interest. We have created a circuit that computes a complex abstract logic (miR1 AND miR2+3 AND NOT miR4 AND NOTmiR5 AND NOT miR6) and selectively triggers output response in HeLa but not in other cells.

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## INTRODUCTION

### Motivation and objective

Modern breast cancer therapies utilize non-specific approaches to kill or remove cancerous cells, inflicting significant collateral damage to healthy cells. In response to the need for highly targeted detection and destruction of cancerous cells, we propose to implement multi-input genetic circuits that act as cell state classifiers based on mRNA or microRNA expression profiling.

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## BODY

### I. Approach

### I.a. mRNA-based cell classifier

In the first approach (Figure 1), the components of our proposed circuit include an apoptotic gene with an engineered regulatory sequence (RS), short interfering RNA (siRNA) or microRNA directed against the RS, and a set of additional short mRNA sequences, mStaples. Each mStaple molecule is complementary to a specific cancer biomarker and partially complementary to a portion of the RS. The role of mStaple is to regulate siRNA mediated degradation of the apoptotic gene. In the absence of mStaple, the RS forms a stem loop where the siRNA binding site is hidden and does not allow for siRNA binding and degradation of the mRNA. As a result, the cell undergoes apoptosis. When the mStaple binds to the RS, it enforces a conformational change of the sequence and exposes siRNA binding site. The mRNA of the apoptotic gene is degraded and the cell survives. The expected behavior is therefore abundance of the mStaple in normal cells and it's shortage in cancer cells. In our system the mStaple is expressed similarly in all cell types, but it's availability for binding of the RS depends on the level of endogenous genes – cancer biomarkers. The mStaple binds preferentially to the biomarker mRNA and with lower affinity to the RS. In normal cells with low biomarker levels some of the mStaple will be bound by the biomarker and some will target the RS to expose the siRNA binding sequence (Figure 1.B). In cancer cells, when the biomarker level is high, the mStaple will be titrated away, causing no disruption in expression of the apoptotic gene, and ultimately cell death (Figure 1A). For testing purposes, we replace the killer gene with a reporter, EYFP gene in the first implementation of the circuit.

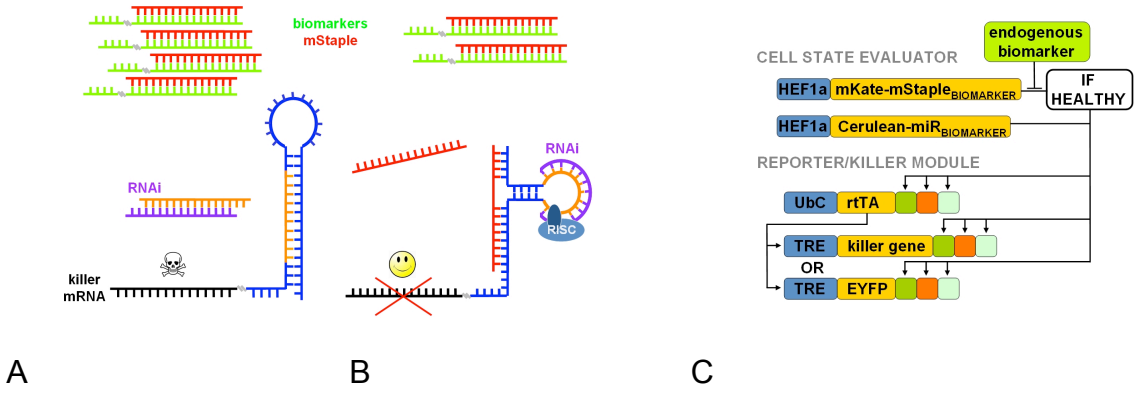



**Figure 1**: RNAi-based logic circuit, approach I. Expression of the apoptotic (killer) protein is dependent on the endogenous biomarker mRNA. Circuit logic in cancer cells (**A**) and normal cells (**B**); circuit implementation (**C**)

### I.b. microRNA-based cell classifier

In our second approach we took advantage of the increasing knowledge of microRNA profiles for different cell types, including various diseased cell states. We have constructed multi-input cell classifier circuit (1) that ascertains whether the expression profile of six endogenous microRNAs matches a predetermined reference profile characteristic of the HeLa cervical cancer cell line. A match identifies the cell as HeLa and triggers apoptosis. The model system can be later customized to recognize and target any cell state of interest, including different types of cancer.

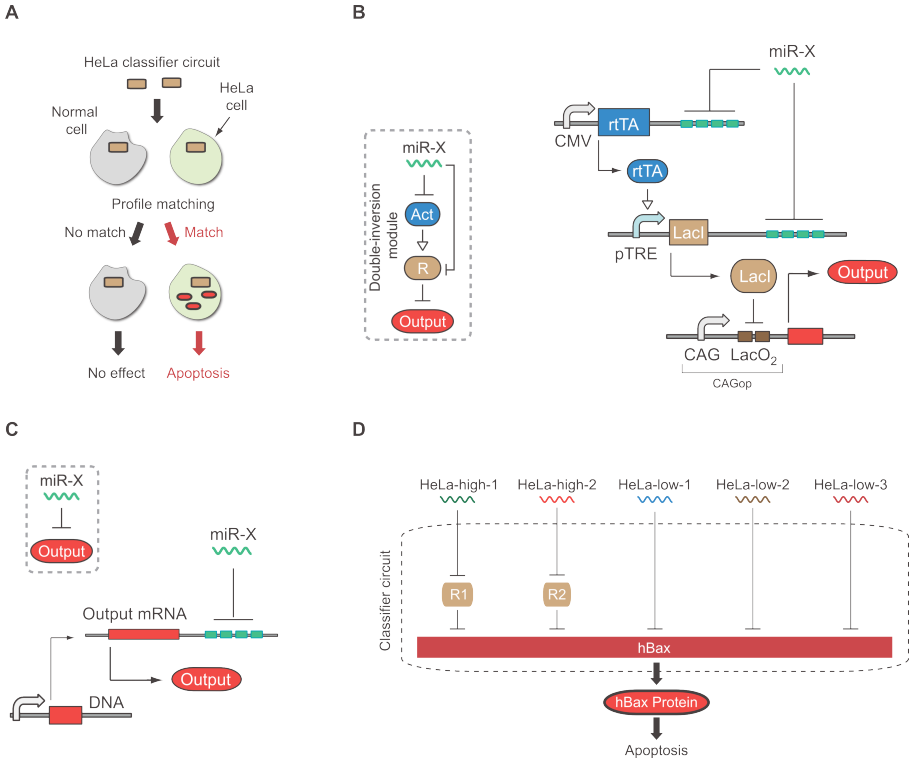



**Figure 2**: Schematic representation of a HeLa-specific classifier circuit operation (**A**). High-level and detailed description of 'double-inversion' module for sensing HeLa-high microRNAs. Act, activator; R, repressor (**B**). High-level and detailed description of HeLa-low microRNA sensor (**C**). Integrated multi-input classifier. R1, R2 - double-inversion modules (**D**). The entire network implements a multi-input AND-like logic function for identification and selective killing of HeLa cells through regulated expression of hBax.

## II. Results

### II.a. mRNA-based cell classifier: regulatory sequence in the 3'UTR hides miRNA binding site and inhibits gene knockdown

We have created and tested all the components of the sensing circuit, using artificial, orthogonal to mammalian expression system and very efficient microRNA targeted against firefly luciferase, miRFF4 (2), and mStaple complementary to Gata3 gene, that is one of the best breast cancer biomarkers.
First, to verify that the designed regulatory sequence is capable of hiding and exposing the microRNA binding site we have tested EYFP-3'UTR knockdown, where the 3'UTR contained one of the following:
(a) 4xFF4, 4 repeats of FF4 target sequence (maximal knockdown),
(b) SSL-FF4, short stem loop (SSL) containing FF4 target site – fragment of RS simulating mStaple bound conformation (knockdown expected),
(c) LSL-FF4, long stem loop (LSL) containing FF4 target site – the full regulatory sequence (knockdown not expected without mStaple present).

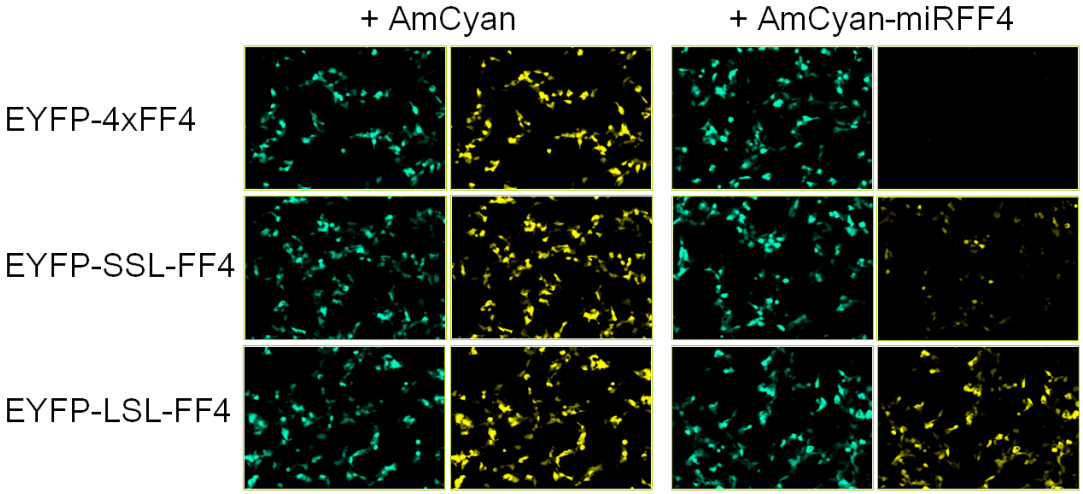



**Figure 3:** 293FT cells were co-transfected with TRE-EYFP-3'UTR and AmCyan or AmCyan-miRFF4 expressing constructs. As designed, the conformation LSL prevents gene knockdown.

The results agree with our predictions very well. The full regulatory sequence hides siRNA target site, whereas the shorter sequence that can only form SSL and therefore simulates the presence of mStaple, exposes the target site (Figure 3).

### II.b. mRNA-based cell classifier: mStaple dependent gene knockdown

In the next step we tested the system with the Gata3 mStaple. We previously showed that the mStaple causes a conformational change of our designed regulatory sequence in vitro. Now we also show that the designed regulatory sequence is capable of the mStaple-regulated conformational change that results in siRNA mediated reporter mRNA degradation in vivo (Figure 3). When a reporter gene with the regulatory sequence in the 3'UTR is co-transfected with either just the targeting siRNA-FF4, or the Gata3 mStaple and a nonsense siRNA, the gene knockdown is lower than in the case of simultaneous co-transfection of siRNA-FF4 and the Gata3 mStaple.

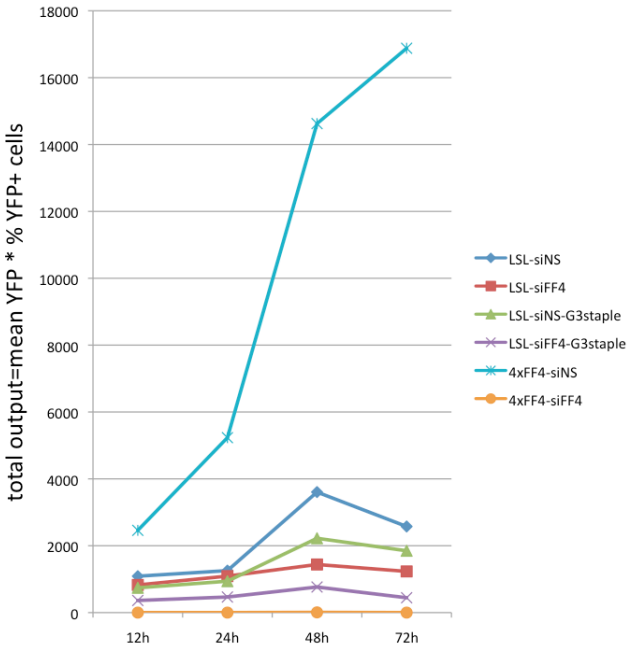



**Figure 3:** Best results for the mRNA sensing sub-circuit; YFP is the actuation gene, LSL (long stem loop) is our designed 3'UTR regulatory sequence, siNS-nonsense siRNA, siFF4-FF4 siRNA, 4xFF4 are 4 repeats of the siFF4 target sequence attached in the 3'UTR instead of LSL (control).

### II.c. microRNA-based cell classifier: the classifier circuit activates output production in HeLa cells only

To test our classifier circuit we performed fluorescence reporter assays in 7 different cell lines. The hBax gene was replaced by fluorescent reporter, DsRed. The circuit correctly identifies HeLa cells among others resulting in the highest output production in HeLa.

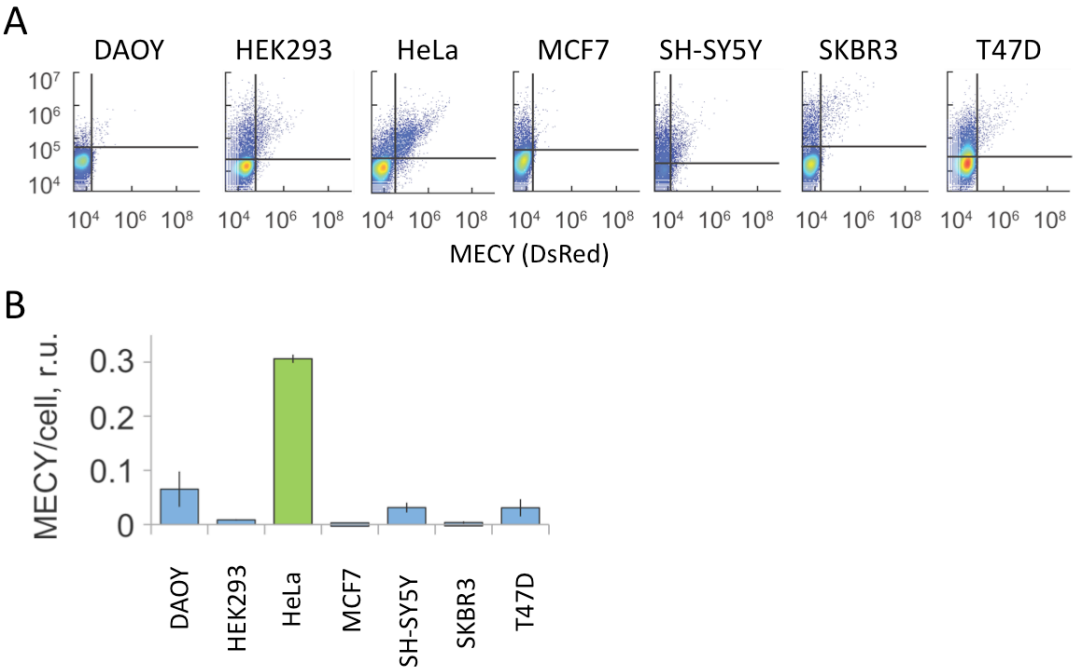
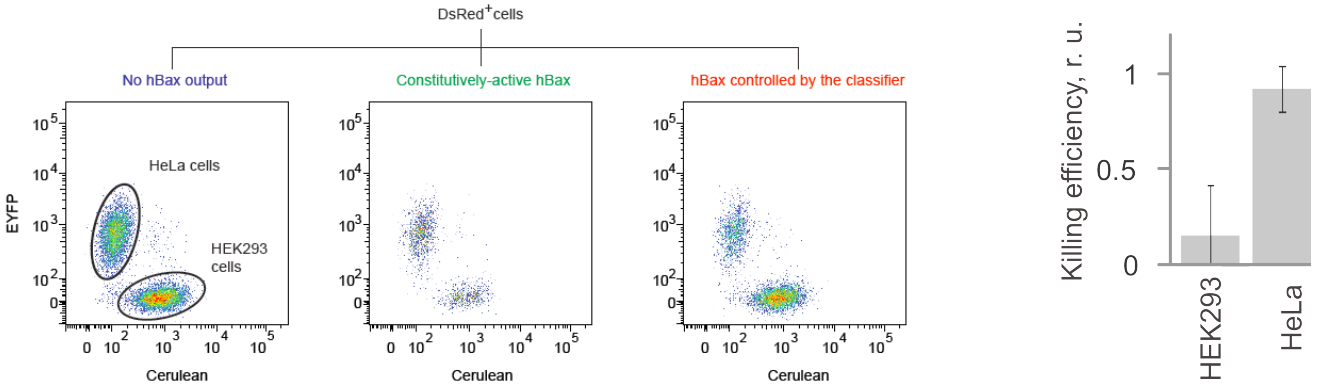



**Figure 4:** Fluorescence reporter assay; representative flow cytometry scatter plots (**A**), relative output levels (**B**). Bars show mean ± SD from at least three independent replicates.

### II.c. microRNA-based cell classifier: in mixed cell culture our circuit identifies and specifically kills HeLa cells

In the next step we replaced the circuit fluorescent output with the apoptotic protein hBax, and tested it's performance in a mixed population of HEK 293 and HeLa cells. When circuit expressing hBax output was transfected into a mixed cell culture, only HeLa cells were selectively killed according to our assay.




A B

**Figure 5:** Apoptosis assay in HeLa and HEK293 cell lines; Mixed culture of Hela cells stably expressing yellow fluorescent protein (FP) and HEK293 cells stably expressing cerulean FP were co-transfected with circuit parts. The resulting fluorescence distribution was compared to a control mixed culture to assess killing efficiency (**A**). Normalized killing efficiency (**B**).

### II.d. microRNA-based cell classifier: Moving towards therapy, single plasmid version of the classifier

Our microRNA cell profiler presents one of the most sophisticated in-vivo biosensors reported to date. Although, many challenges still need to be overcome before this or other synthetic biology circuits can be used in the clinic. Moving the work forward, we chose to focus on improving the implementation of the classifier to make it simpler for delivery and more suitable for future possible applications (therapy). Additionally we studied microRNA profiles of other diseased cell states with the aim of creating multiple therapy-relevant classifier circuits.

In the first implementation, the profiler circuit was delivered in co-transfection of 5 plasmids and the 6-th plasmid was used as a transfection control (Figure 6, blue shade). This configuration is far from optimal for possible applications, given that all the parts need to be present in order for the classifier to be fully functional. In the first approach we created and tested a single-plasmid version of the classifier circuit using an assembly method, newly developed in our lab (3). In short, promoters, genes and the appropriate 3'UTR regulatory sequences are recombined using standard Gateway cloning (4) and inserted in a specially designed destination vector to create one transcription unit (TU) position vectors. In the second step the position vectors are assembled together with a carrier vector (new backbone) in a single Gibson assembly reaction (5). During the assembly process the backbones of position vectors are disregarded, and the final vector contains TU1-TU2-…-TUx. Additionally, each position vector contributes a chromatin insulator upstream of the TU (cSH4), to prevent interference between the parts.

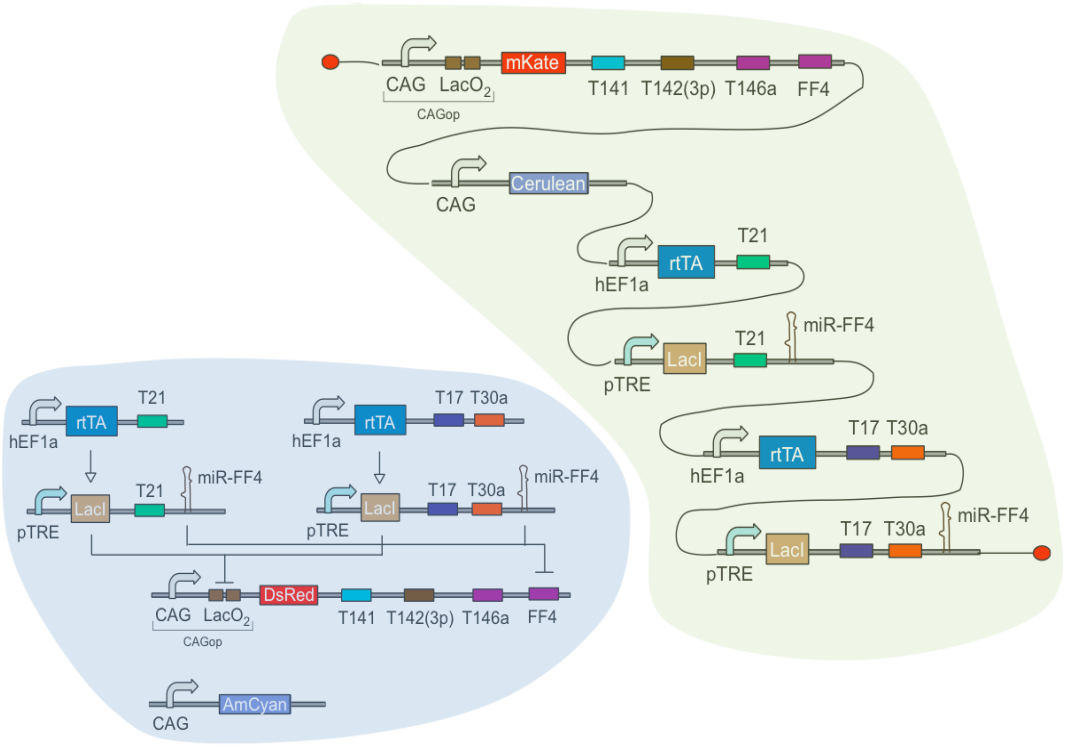



**Figure 6**: microRNA-based HeLa classifier circuit in a multi-plasmid (gray shade) and single plasmid (green shade) configuration.

We have tested performance of the single-plasmid version of our microRNA classifier circuit by transient transfection in HEK 293 and HeLa cells. When compared with the multi-plasmid co-transfection data (Figure 7) the results are very encouraging. Single-plasmid classifier performs at least as good as the original circuit version with a slightly lower false positive rate (lower HEK293/HeLa overlap for the circuit controlled output).

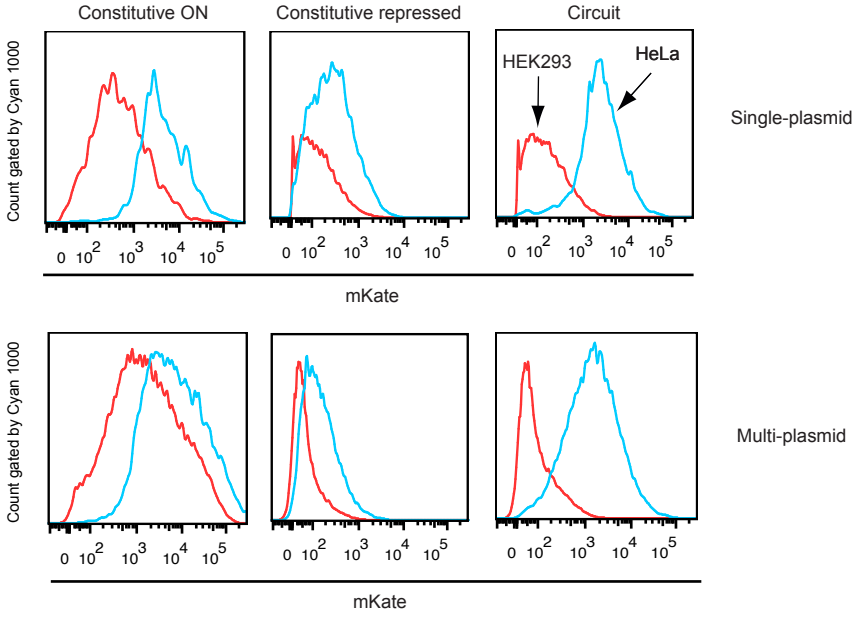



**Figure 7:** Performance of the microRNA classifier in single-plasmid configuration, as compared with multi-plasmid co-transfection: representative FACS histograms.

**III. Outreach**

The following students were involved in the project and mentored:
Genia Dubrovsky (junior project, 2009)
Anna Igorevna Podgornaia (rotation student, 2009)
Hattie Chung (Amgen scholar, 2010)

## KEY RESEARCH ACCOMPLISHMENTS

mRNA-based classifier circuit:

- Design and experimental verification of the 3'UTR regulatory sequences SSL and LSL that can expose or hide microRNA target site regulating gene knockdown;
- Demonstration of gene knockdown regulation by a short RNA oligunucleotide, mStaple.

microRNA-based classifier circuit:

- Design and experimental verification of a sophisticated multi-input microRNA-based cell classifier circuit that can specifically activate output production in HeLa;
- Demonstration of specific killing of HeLa cells when a mixed culture is transfected with the classifier circuit that controls production of a pro-apoptotic gene hBax.
- Development and testing of a single-plasmid version of the microRNA classifier circuit as a step forward in the direction towards its therapeutical application.

## REPORTABLE OUTCOMES

The work has been presented on the following conferences:

- The American Society for Cell Biology annual meeting, Philadelphia, PA, Dec 11-15, 2010
- SB5.0: The Fifth International Meeting on Synthetic Biology, Stanford, CA, Jun 14-17, 2011
- Era of Hope, Orlando, FL, Aug 2-5, 2011

The microRNA classifier work have been published:

Xie, Z., Wroblewska, L., Prochazka, L., Weiss, R., and Benenson, Y. (2011). Multi-input RNAi-based logic circuit for identification of specific cancer cells. *Science* 333, 1307-1311.

## CONCLUSION

We have predicted and demonstrated experimentally that microRNA dependent gene knockdown can be regulated by 3'UTR secondary structure of the target gene. The secondary structure can be changed with the help of exogenously added short RNA oligonucleotide, mStaple. Based on that, we created an mRNA sensing circuit that shows promising results in a fluorescent assay, although still requires further optimization.
We have also designed and successfully implemented microRNA-based classifier circuit for selective detection and destruction of cancer cells. Our classifier identifies and kills HeLa cells among other cell lines by compiling information about levels of specific microRNAs in the cell and triggering production of the output (fluorescent or pro-apoptotic protein) only when a certain microRNA profile is matched.

Building on new circuit assembly technologies, we have created a single plasmid version of the classifier circuit. The new circuit configuration did not influence its performance and made it more manageable for future possible applications (therapy).

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## APPENDICES